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THE STORY OF VITAMIN B_{12}

By RUTH WOODS

Vitamin B_{12} was discovered last Spring in April, 1948. Its story, however, began more than twenty years ago with the discovery that removed pernicious anemia from the list of inevitably fatal diseases of mankind. That discovery was that pernicious anemia could be cured by simple dietary means —the eating of whole liver. At least a pound a day. The cure, miraculous though it was, was indeed a formidable one. Liver once a week in an average serving of four ounces is a commendable dietary practice for the average normal adult. Liver seven days a week in a total of twenty-eight four ounce servings must be considered a heroic, if not herculean, achievement.

During the years that followed, chemists succeeded in extracting from liver those portions which were largely responsible for the anti-pernicious anemia effects. By 1943, these extracts were so far concentrated that patients could now survive by taking only one milligram of liver extract instead of 400,000 milligrams of liver. Of this one milligram, only about .005 to .010 mg. (i.e. 5-10 micrograms) is responsible for the curative properties. This minute fraction is vitamin B_{12}, the material isolated last April after decades of painstaking research.

The story of vitamin B_{12} is interwoven with that of folic acid; with the discovery of a new vitamin necessary for baby rats to grow and for chickens' eggs to hatch; and with many other apparently irrelevant chapters in scientific investigation. It is a story of the combined achievements made possible through research in medicine, chemistry, microbiology and nutrition.

The Search for an Anti-Pernicious Anemia Factor:

Early History: Pernicious anemia was recognized as a clinical entity one hundred years ago by Addison (1). It is a type of anemia* characterized by a reduced number of red blood cells of abnormally high hemoglobin content. The red blood cells themselves are immature, large forms. Other characteristic abnormalities of the blood are present also, as is the closely related disturbance *achylia gastrica* (a wasting of the gastric mucosa with consequent absence of both the digestive enzyme, pepsin, and hydrochloric acid from the gastric secretions). Changes in the central nervous system are also frequently associated with pernicious anemia.

**cf. Borden's Review of Nutrition Research,* March, 1946, "Nutritional Milestones in the Anemias."

Up until 1926 various treatments were tried with little success. Most of them were based largely on the belief that pernicious anemia was a disease of infectious, toxic or neoplastic nature. It was thought that the abnormal blood picture was the result of pathological destruction of the red blood cells rather than, as is recognized today, the result of defective red cell formation.

The first inkling that these early beliefs might not be valid came in 1925. At that time a British investigator, Elders (2), pointed out the similarity between tropical sprue and anemia. He believed sprue to be a deficiency disease, having successfully treated it since 1916 by dietary means. With the observation that a case of pernicious anemia responded favorably to the diet used in the treatment of sprue,* Elder suggested that pernicious anemia is also a deficiency disease.

In the same year, Whipple and coworkers (3) in the United States working with experimentally induced anemia in dogs, reported the favorable influence of beef liver in enabling the body to regenerate new blood cells. Although the anemia in dogs, produced by repeated hemorrhages, was not the same as pernicious anemia, they concluded that food factors such as might be present in liver might be of considerable value in the therapy of human anemias as well.

The following year, Koessler *et al* (4) also reported the importance of diet in the cause and treatment of human anemias and classified the disease as a manifestation of vitamin deficiency. Although concerned primarily with providing vitamins A, C and the B-complex, their diet included liver and kidney.

Liver as a Specific Anti-Pernicious Anemia Agent:

Coincident with the vitamin deficiency theory of Koessler *et al,* the same issue of the Journal of the American Medical Association carried the epoch-making report by Minot and Murphy (5) on the effectiveness of whole liver therapy in the clinical treatment of human pernicious anemia. They reported in this and in a later study (6) a prompt and distinct improvement in a large number of pernicious anemia patients on a diet in which liver was a major constituent. Within two to eight days of receiving such a diet, a marked increase in reticulocytes** was obtained. With continued liver therapy, the increase in reticulocytes was followed by a rise in hemoglobin and total

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*The diet consisted of underdone meat up to 1 kg. or more *(2.2 lbs. or more);* with 1½ to 2 liters of raw milk *(a little over 1½ to 2 qts.)* plus calcium supplements, cod liver oil, a few oranges or 400 g. (1 lb.) of strawberries per day.

**Type of networked red blood cell which occurs when active blood regeneration is going on.

red cell count and a return to an approximately normal blood picture in about two months. Associated with the improvement in the blood condition were a rapid gain in weight and a sudden improvement in general mental outlook.

The findings of Minot and Murphy led to the inevitable conclusion that an active anti-pernicious anemia factor exists in liver. The anti-pernicious anemia factor is generally believed to be a factor combining two unidentified principles described by Castle,* the *intrinsic* factor (secreted by certain stomach glands) and the *extrinsic* factor (an essential complementary factor found in meat, eggs, yeast and liver). Either of these alone is incapable of effecting a cure in pernicious anemia. Liver provides a complete anti-anemia factor which may be the combined intrinsic-extrinsic factor or perhaps another substance altogether. At any rate, the liver factor will cure anemia regardless of the presence or absence of the intrinsic factor in the patient.

Chemistry of the Liver Factor: From 1926 until 1948, chemists all over the world concentrated their efforts in an attempt to isolate and identify the active principle in liver responsible for the remarkable cures in pernicious anemia patients. Liver was ground up, dissolved in water and other solvents, filtered, precipitated, extracted, dialyzed and otherwise fractionated into as many conceivable component parts as possible. Each of the liver fractions thus obtained (and there were literally thousands) had to be tested for anti-pernicious anemia therapy by the only procedure available: Clinical trial with a patient suffering from pernicious anemia. Because of this fact, tests of the innumerable liver fractions obtained by chemists proceeded at a hopelessly slow rate dependent upon the availability of pernicious anemia patients for experimentation. The painstaking studies on the fractionation of liver; the discovery of apparently widely varying types of fractions with demonstrated anti-pernicious anemia activity; and the step by step elimination of the known characteristics of these fractions (i.e. proteins, alpha amino acids, carbohydrate, etc.) as the active principle were reviewed with great thoroughness by Subbarow, Hastings and Elkin (7) in 1945. By that time, a considerable number of relatively highly concentrated liver preparations had been developed by chemists and were all being used successfully in the clinical treatment of pernicious anemia. Subbarow *et al* pointed out the great variation existing in the chemical properties of the numerous liver preparations to which activity had been attributed, stating:

> "The reasonable explanation for these differences is that the various preparations are still more or less contaminated and the activity of the different products

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**cf. Borden's Review of Nutrition Research,* March, 1946, "Nutritional Milestones in the Anemias."

may be due to one unknown substance which is present in all these products in small amounts. However, the chemical properties of these various materials vary with the nature of the impurities in the various preparations."

Although nearly two decades had elapsed since the introduction of whole liver therapy and the review by Subbarow and coworkers without the exact identity of the anti-pernicious anemia factor in liver having been uncovered by any of the investigators of this problem, the authors pointed out that research during this period culminated in at least one significant fact: *"The amount of material needed by the patient per day has decreased from 400 g. to less than 1 mg."** They concluded with the prophetic statement that such progress made it reasonable to expect the isolation and identification of the active material to be an attainable objective. Such was the status of the anti-pernicious anemia factor as late as 1945.

A New Contribution of Nutrition Research:

Leaving for the moment the exciting nineteen year old hunt which held great promise of closing in on the elusive nature of the anti-pernicious anemia substance in liver, our story shifts to an entirely new field of investigation. The workers in this field were concerned primarily with extending existing knowledge concerning the nutritive requirements of animals and, ultimately, of man. They had, at the beginning of their studies no interest in the anemia problem nor any premonition that their investigations would eventually merge with those of the anemia researchers.

A New Vitamin Factor is Discovered: In 1946, Cary, Hartman and associates (8) reported that rats on a diet adequate in all known nutrients require a new, unidentified factor essential for normal growth. The new factor, tentatively termed X, was found to be stored normally in young rats at weaning. By feeding mother rats a diet deficient in factor X, however, it was possible to obtain young animals depleted of this factor. Such depleted animals showed marked retardation of growth and, in cases of severe depletion, generally died within two weeks. Casein prepared by simple centrifugation from milk was found to be a good source of X; commercial "vitamin-free" caseins were variable sources; while casein prepared by repeated hot alcohol extraction was found to be almost devoid of X. Animals on adequate diets, but whose stores of X had been depleted by maternal undernutrition, failed to grow normally when fed 20% or 40% of alcohol extracted casein. Normal growth was restored when small amounts of a commercial liver extract were fed, thus indicating that liver provided a potent source of X.

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*In other words, a decrease of 400,000-fold from approximately one pound of liver per day to about .0003 oz. of a liver extract.

Soon afterward, Hartman and Cary (9) reported further studies on the occurrence of X in various foods besides casein and certain liver extracts. Using X-depleted young, the growth-promoting activity of a number of foods was tested. The test foods were fed in such a way as not to disturb the completeness of the rations as sources of known nutrients. Certain foods produced normal growth; others increased growth with increasing doses. Such foods were milk, powdered skim milk, cheese, beef and pork muscle, egg yolk and certain leafy foods and feedstuffs. The potency of egg yolk appeared to vary with the diet of the hen, indicating transmission of the factor from the hen to the chick through the egg.*

Similar studies with closely related, if not identical, factors were being conducted by numerous other investigators in poultry nutrition, both prior to and during the work on the rat factor. The earliest of these was a report by Parkhurst (9a) in 1927, just one year following the discovery of the role of liver in pernicious anemia. He reported that normal hatchability of eggs could not be obtained unless breeding rations contained some protein of animal origin. The findings of these combined researches led eventually to the postulation of the new "animal protein factor."*

Possible Relation to Anemia: While the studies with rats and poultry were going on, another approach to the identification of factor X was made through microbiological studies. The following year, Shorb (10) reported an attempt to find a microorganism that might require the rat growth factor described by Cary and Hartman. As a result it was noted that the organism, *Lactobacillus lactis* Dorner required the presence of two unidentified factors for growth in addition to all the known synthetic B vitamins and amino acids. One factor was found to be present chiefly in tomato juice (TJ factor) and, in small amounts, in casein. The other heat-stable factor required by the *Lactobacillus lactis* Dorner (LLD factor) was found in highest concentrations in the liver extracts reported active for rat growth, but not in casein or casein hydrolysates.

Assays for the LLD factor were made using crude and refined liver extracts of the type used for intramuscular injection in the treatment of pernicious anemia. Results showed that the LLD factor is apparently concentrated in the refined extracts in almost linear relationship to the potency of these extracts for effecting cures in pernicious anemia (11). Shorb stated that this relationship suggested the possibility that the LLD factor might be the active principle in pernicious anemia.

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*cf. *Borden's Review of Nutrition Research,* October, 1948, "The Animal Protein Factor."

Isolation of the Anti-Pernicious Anemia Factor:

While all of the biological and microbiological studies were going on in the search for a new nutritive essential, the "animal protein factor," the search begun by chemists in 1926 for the anti-pernicious anemia factor in liver was being conducted as vigorously as ever. A brief surge of hope that this elusive factor had finally been found arose when folic acid was discovered and isolated in pure crystalline form.* This hope was short-lived, however. Clinical studies showed that although positive hematological responses are obtained with folic acid in some cases of pernicious anemia, the neurologic symptoms of this disease either fail to respond or may even become more severe with folic acid therapy (12, 13). Further, folic acid is not always effective in pernicious anemia, while liver extracts are; also, active liver extracts do not necessarily contain significant amounts of folic acid in any of its biologically effective forms.

Vitamin B_{12}: The climax to this hectic search came on April 16, 1948 with the announcement of Rickes *et al* (14) that biologically active, pure, crystalline material had at last been isolated from clinically active liver concentrates. The crystalline material, tentatively named vitamin B_{12}, was found to exert LLD factor activity for the *Lactobacillus lactis* Dorner (15), thereby confirming the earlier suggestion by Shorb (11) of the identity of these two materials. Finally, clinical studies with crystalline vitamin B_{12} proved its effectiveness in inducing favorable blood responses in pernicious anemia (16). The following week, on April 24, the purification of two amorphous forms of the anti-pernicious anemia factor from liver was announced independently by Smith (17) in the British journal, Lancet. The product, though not as pure as the crystalline B_{12} of Rickes *et al* (14) exhibited highly similar physical and chemical properties. Unpublished clinical studies by Ungley in England (cited by Smith) revealed the amorphous material to be effective not only in stimulating blood regeneration, but also in the treatment of three cases of subacute combined degeneration of the spinal cord associated with pernicious anemia.

Three months later, Stokstad and associates (17a) announced the isolation of a crude material having high animal protein factor activity as measured by assay with chicks. The material was found to be synthesized by a non-motile, rod-shaped organism isolated from hen feces. When tested on human patients with pernicious anemia it was found to stimulate a favorable hematopoietic response.

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*cf. *Borden's Review of Nutrition Research,* February, 1946, "Folic Acid."

In a continuation of their studies, Rickes *et al* (17b) investigated the possible occurrence of the new vitamin in biological materials other than liver. Several materials were found to exhibit growth activity for *L. lactis* Dorner, including milk powder, beef extract and culture broths of various microorganisms. Of these active materials, one—a red crystalline compound isolated from the mold, *Streptomyces griseus* (producer of streptomycin)—has been shown to have physical and chemical properties identical with those of crystalline vitamin B_{12} from liver. Unpublished clinical tests with the material produced by *S. griseus* have shown responses in pernicious anemia comparable to those obtained with vitamin B_{12}. From these considerations it is concluded that the crystals from the microbiological source and vitamin B_{12} from liver are identical.

The synthesis of vitamins B_{12} by the *Streptomyces* organism, in much the same manner as the antibiotics streptomycin and penicillin are produced, may afford a more convenient and less expensive method of obtaining the new vitamin. The significance of this possibility may be readily appreciated when it is remembered that a ton of fresh, raw liver is required in order to yield the infinitesmal quantity of about 10 to 20 milligrams of crystalline vitamin B_{12}.

Chemistry of Vitamin B_{12}: Vitamin B_{12} crystallizes in the form of small red needles. Smith (18) in England reported the presence of both cobalt and phosphorus in the crystalline material, a finding which was simultaneously confirmed by Rickes *et al* (19) in this country. Some nitrogen, but no sulfur has also been detected in the compound, the probable molecular weight of which has been estimated to be between 1550 and 1750. The most significant finding to date has been the presence of cobalt, heretofore unknown as a component of a natural organic compound. Cobalt, as a trace inorganic element, has long been recognized as a nutritional essential in ruminants in whom cobalt deficiency results in a typical anemia (20). The significance of trace amounts of cobalt in the therapy of iron deficiency anemias in man is also well known (21).

Biochemical Activity of Vitamin B_{12}:

Potency: Vitamin B_{12} has been found to be biologically active in extremely minute quantities. Compared to an arbitrarily selected standard liver concentrate active for *Lactobacillus lactis* Dorner having an assigned potency of 1000 LLD units per milligram, vitamin B_{12} has a potency of about *11,000,000 LLD units per milligram* (15). Only 0.000013 *microgram* per ml. of

culture medium is required for half-maximal growth of the microorganism. This places vitamin B_{12} among the most potent of microbiologically active compounds.

Vitamin B_{12} is apparently of similar high potency in the clinical treatment of anemia. Doses of 3, 6 and 150 micrograms have been found highly effective (16). Comparable effects with folic acid have required dosages of from 20,000 to 50,000 micrograms during the first ten days of treatment (22, 23).

A comparison of the activity of U.S.P. liver concentrates and of vitamin B_{12} was made by Rickes *et al* (14) in an attempt to demonstrate that the vitamin B_{12} content of such concentrates may be considered to account entirely for their activity. According to their calculations:

> "One U.S.P. unit is defined as that amount of liver extract required daily to produce satisfactory clinical and hematological responses in Addisonian pernicious anemia. If it is assumed that this new crystalline compound is the only substance present in these preparations which is therapeutically active, it is evident that clinical response should be obtained from the parental administration of approximately 1 microgram of the new vitamin per day. The clinical responses obtained with single 3- and 6-microgram doses of crystalline vitamin B_{12} are not inconsistent with the approximate equivalence of 1 microgram of the vitamin and 1 U.S.P. injectable unit. It should be pointed out, however, that it is customary to administer 20-60 U.S.P. units of liver extract during the first two or three days to start remission of pernicious anemia in relapse. This dose range is equivalent to not more than about 20-60 micrograms of vitamin B_{12}."

Relation to Animal Protein Factor: As has already been indicated, vitamin B_{12} and the LLD factor are probably identical. The LLD factor, in turn, is either closely related to or identical with the "animal protein factor" required for rat and chick growth and for proper hatchability of chicks. The close relationship or possible identity of vitamin B_{12} with the "animal protein factor" is, of course, thereby also implied. More conclusive evidence in this direction, however, has been afforded by the recent work of Ott *et al* (24). In July, 1948 they reported that crystalline vitamin B_{12}, when added to purified basal diets with 40-70% soybean meal as the only source of protein, exerted animal protein factor activity in chicks from hens on all-plant ration such as described by Rubin and Bird (25). Chicks responded to various supplements known to be sources of the animal protein factor and also to vitamin B_{12} in doses as small as 6 micrograms per kilo of diet. The authors concluded that since the crystalline substance elicited growth responses comparable to crude sources of the animal protein factor, it is possible that vitamin B_{12} is identical with or closely related to this factor.

Relation to Folic Acid, Thymine and Thymidine: During 1948 a crystalline factor functionally related to folic acid was isolated from liver (26). This compound has been identified as thymidine (the desoxyriboside of thymine*). Thymine itself had previously been reported to be able to substitute for folic acid (in a ratio of 5000:1) in the nutrition of lactic acid bacteria (27). In one case, thymine in large doses was found to replace folic acid in the treatment of human macrocytic anemia (28). More recently, the related compound, thymidine, has been found to be capable of replacing vitamin B_{12} in the nutrition of *Lactobacillus lactis* (29, 30). (Thymine, however, does not replace B_{12}). Because of the large amounts of thymidine required to replace B_{12}, the two are not considered identical. Wright *et al* (30) have interpreted the data to indicate that vitamin B_{12} acts as a coenzyme in the processes involved in the conversion of thymine to thymidine. They postulate further that the primary biochemical defect in pernicious anemia may well be the inability to synthesize certain nucleosides,** particularly thymidine, from parent purines or pyrimidines. They, therefore, attribute the curative effects of folic acid in some cases of pernicious anemia to increased thymine synthesis (27), as confirmed by the clinical effectiveness of thymine as a substitute for folic acid therapy (28). The increased thymine resulting either from heightened synthesis (due to folic acid) or from actual therapeutic administration, in turn, is then believed to yield, by mass action, more of the essential thymidine. Still further interrelations among vitamin B_{12}, thymine, thymidine, purines, folic acid and the animal protein factor are discussed in an interesting note by Shive *et al* (31) published just two months ago (November, 1948). They confirm the postulated role of B_{12} in the biosynthesis of thymine and thymidine and indicate also its functional identity with the animal protein factor in this activity.

Clinical Use of Vitamin B_{12}:

At the present time there have been published reports of only a few cases of pernicious anemia treated with pure vitamin B_{12} (16, 17, 32, 32a). The first three of these were reported by West (16). These received 150, 6 and 3 micrograms of the vitamin, respectively in a single dose, injected intramuscularly. Reticulocytes, red blood cells and hemoglobin increased immediately

*A pyrimidine compound, not be confused with the vitamin, thiamine.

**The nucleosides are integral parts of the nucleoproteins, which, in turn, are important structural constituents of cells. It is apparent how their synthesis may be involved not only in the formation of new red blood cells, but also in the apparently unrelated phenomenon of chick hatchability—another phase of new cell and tissue formation.

following the single dose in each patient. The question of whether the blood picture will return to normal must await further study of the patients and, possibly, continued treatment with the vitamin.

In the cases reported by West no mention was made of the effect, if any, of vitamin B_{12} on the neurological symptoms of pernicious anemia. Although Smith (17) had mentioned unpublished results indicating the successful response of three cases of combined system disease (spinal column degeneration) to therapy with purified (amorphous) anti-pernicious anemia factors, the material used was less pure than crystalline vitamin B_{12}. Therefore, although British and American investigators were both working with the same essential substance, it was possible that an impurity still remaining in the amorphous British product might have been responsible for the neurological cures obtained.

This doubt has apparently been eliminated by a recent case report of Berk *et al* (32). These physicians treated a pernicious anemia patient with severe neurological symptoms with 5 micrograms of B_{12} per day for eight days. By the fourth day, a rise in reticulocytes was obtained, reaching a peak on the sixth day. A 58 per cent increase in red cells and hemoglobin was reached in 10 days. After 10 days remarkable changes in the neurological disorder were already evident. Feelings of numbness had already vanished by the fourth day; by the tenth day, reflex responses had returned to normal, unsteadiness was gone and the patient could walk unsupported. When administration of vitamin B_{12} was stopped, the neurological symptoms began to reappear. Resumption of vitamin B_{12} therapy again resulted in neurologic improvement, thereby establishing vitamin B_{12} as being responsible for the observed neurologic improvement.

From the limited amount of clinical evidence that has become available in the few months following the isolation of pure vitamin B_{12}, there is little doubt that this substance is indeed the long sought anti-pernicious anemia factor. Many more clinical studies and more complete evaluations of the efficacy of vitamin B_{12} will have to be made over greater periods of time, however, before this assumption can be made without doubt or qualification.

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